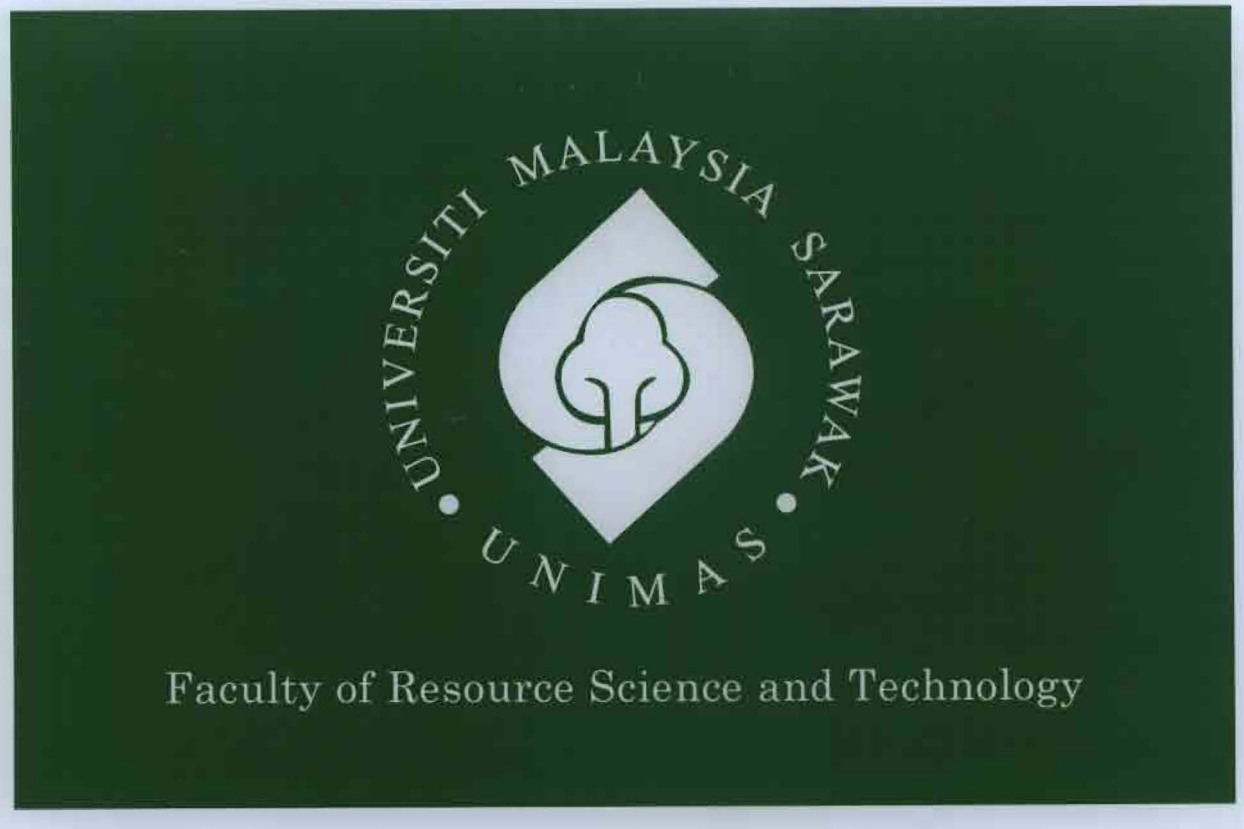

SCREENING, ISOLATION AND CHARACTERIZATION OF PHENOLIC COMPOUND PRODUCING FUNGI USING AGRICULTURAL WASTE AS SUBSTRATE

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DECLARATION

I hereby declare that this thesis is based on my original work except for quotation and citation, which have been duty acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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LIST OF ABBREVIATIONS

°C	degree Celcius
%	percentage
M	molar
ml	milimiter
rpm	revolution per minutes
sp.	Species
SSF	Solid-state fermentation
mg	milligram
mg/ml	milligram per milliter
PDA	Potato Dextrose Agar
ITS	Internal Transcribe Sequences

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ABSTRACT

This research focuses on the characterization of fungi producing phenolic compound which is ferulic acid through solid-state fermentation using rice husk and empty fruit bunch as substrates. Screening of isolated fungi by using total phenolic content and feruloyl esterase assays showed that UMAS P2 and UMAS B2 were able to produce phenolic compound which is ferulic acid. The morphological characteristic and molecular identification was done and the result showed that UMAS P2 is *Rhizomucor variabilis* and UMAS B2 is *Fusarium chlamydosporum*. Determination of the production of phenolic compound using *Rhizomucor variabilis* and *Fusarium chlamydosporum* under solid state fermentation is achieved by total phenolic content, while feruloyl esterase assays for the detection of ferulic acid. The production of phenolic compound by *Rhizomucor variabilis* under solid state fermentation of rice husk and empty fruit bunch was recorded as 0.087 mg/g and 0.156 mg/g, respectively, while the production of phenolic compound by *Fusarium chlamydosporum* under solid state fermentation of rice husk and empty fruit bunch was 0.078 mg/g and 0.065 mg/g respectively. Besides, the feruloyl esterase activity by *Rhizomucor variabilis* under solid state fermentation of rice husk and empty fruit bunch was recorded as 1.721 U/g and 0.597 U/g, while the feruloyl esterase activity by *Fusarium chlamydosporum* under solid state fermentation of rice husk and empty fruit bunch was 1.806 U/g and 1.024 U/g, respectively. In conclusion, *Rhizomucor variabilis* and *Fusarium chlamydosporum* have an ability to produce phenolic compound by utilizing agricultural waste such as rice husk and empty fruit bunch as carbon source.

Key words: Phenolic compound, solid state fermentation, total phenolic content

ABSTRAK

Kajian ini memberi tumpuan kepada pencirian kulat menghasilkan sebatian fenolik iaitu asid ferulik melalui fermentasi berkeadaan pepejal menggunakan sekam padi dan tandan buah kosong sebagai substrat. Saringan kulat terpencil dengan menggunakan jumlah kandungan fenolik dan esei 'feruloyl esterase' menunjukkan UMAS P2 dan UMAS B2 dapat menghasilkan sebatian fenolik iaitu asid ferulik. Pengenalan ciri-ciri morfologi dan molekul telah dilakukan dan hasilnya menunjukkan bahawa UMAS P2 adalah Rhizomucor variabilis dan UMAS B2 adalah Fusarium chlamydosporum. Penentuan pengeluaran sebatian fenolik menggunakan Rhizomucor variabilis dan Fusarium chlamydosporumunder fermentasi berkeadaan pepejal dicapai dengan jumlah kandungan fenolik, manakala esei 'feruloyl esterase' untuk mengesan asid ferulik. Pengeluaran sebatian fenolik oleh Rhizomucor variabilis bawah fermentasi berkeadaan pepejal sekam padi dan buah tandan kosong dicatatkan sebagai 0.087 mg/g dan 0.156 mg/g, manakala pengeluaran sebatian fenolik oleh Fusarium chlamydosporum bawah fermentasi berkeadaan pepejal sekam padi dan buah tandan kosong adalah masing-masing 0.078 mg/g dan 0.065 mg/g. Selain itu, aktiviti 'feruloyl esterase' oleh Rhizomucor variabilis bawah penapaian berkeadaan pepejal sekam padi dan buah tandan kosong dicatatkan sebagai 1.721 U/g dan 0.597 U/g, manakala aktiviti 'feruloyl esterase' oleh Fusarium chlamydosporum bawah fermentasi berkeadaan pepejal sekam padi dan tandan kosong adalah 1,806 U / g dan 1,024 U / g, masing-masing. Kesimpulannya, Rhizomucor variabilis dan Fusarium chlamydosporum mempunyai keupayaan untuk menghasilkan sebatian fenolik dengan menggunakan bahan buangan pertanian seperti sekam padi dan tandan buah kosong sebagai sumber karbon.

Kata kunci: *sebatian fenolik, fermentasi berkeadaan pepejal, jumlah kandungan fenolik*

1.0 INTRODUCTION

A microorganism is small creatures that are important in maintaining life on earth. They are classified into various groups which are bacteria, fungi, protozoa, micro-algae and viruses. These microorganisms live in variety of habitats such as soil, water, and animal intestines (Mosttafiz, 2012). Nowadays, microorganism has been extensively used in food industry, textile, agricultural and fermentation process. Some of the microorganisms are used in the manufacturing of fermented foods such as bread, yogurt, and cheese.

In this modern era, microorganisms such as fungi are extensively used in the production of valuable products. One of the valuable products that can be produce by fungi is phenolic compound. Phenolic compound is a secondary metabolite that can be classified into non-soluble compound and soluble compound. Non-soluble compound consist of tannins, lignin and cell wall bound hydroxycinammic acids while soluble compound consist of flavonoid, phenolic acids, phenylpropanoid and quinones (Rispail et al., 2005).

Phenolic compound are highly required in pharmaceutical industry because of their potential ability in supplementary medicinal application that support human health. Recently, the demand of these bioactive compound have been increases due to their beneficial features that able to promote antioxidant, anticancer, antiviral, anti-mutagenic, anti-allergenic, anti-inflammatory, and anti-microbial (Huang, Cai, & Zhang, 2009). Ramamoorthy and Bono (2007) found that most of neurodegenerative diseases, heart diseases, cancer and aging process are caused by oxidative stress. The demand for phenolic compound has extremely increased due to their high antioxidant activity that able to reduce oxidative damage and prevent the development of chronic, age-related degenerative diseases.

Nowadays, large quantities of agricultural waste such as straw, bagasse, cobs, and husks have been produced worldwide. A proper management of agricultural waste is required because improper management of these wastes will lead to many environmental risks and potential diseases to human. As proper management of agricultural waste are costly, scientists have discovered an alternative ways to reduce the amount of agricultural waste by employ it as substrates in the fermentation by using fungi.

The use of agricultural wastes as a substrate for production of valuable products such as secondary metabolites, enzymes, organic acids, and aroma compounds through solid state fermentation is economically crucial and can reduce many environmental risks (Pandey, 2003).

1.1 OBJECTIVES

This research work was conducted to achieve several objectives as stated below:

i. To screen, identify and characterize phenolic compound producing fungi.
ii. To employ agricultural waste as a carbon source for the fungi to produce phenolic compound under SSF.
iii. To quantify the amount of ferulic acid being produced by the selected fungi using different types of substrates.

2.0 LITERATURE REVIEW

2.1 Fungi isolated from rotten fruit

Fungi play an important role in the rotting of fruit. So, different species of fungi can be isolated from different types of rotten fruit such as banana, mango, lemon, oranges, and pineapples. Based on studies conducted by Bashar and co-worker (2012), about 11 fungi species are isolated from rotten mango and *Aspergillus niger* was determined as the predominating fungi in mango rot. According to Al-Hindi, Al-Najada, and Mohamed (2011), different species of fungi which are *Fusarium oxysporum, Aspergillus oryzae, Aspergillus awamori,* and *Aspergillus flavus* can be isolated from rotten banana, oranges, lemon, and mango, respectively. From 2008 until 2011, about 43 *Fusarium* isolates were cultured from 90 banana samples collected from fruit markets and banana orchards in South China. 10 of them were recognized as *F. oxysporum*, *F. solani*, *F. camptoceras*, *F. pallidoroseum*, *F.stiloides*, *F. chlamydosporum*, *F. verticillioides*, *F. proliferatum*, *F. concentricum*, and *F. Sacchari* (Zeng et al., 2013). According to Baffi and co-workers (2012), 14 fungal species were isolated from the olive fruit belonging to 7 different genera which are *Aspergillus, Penicillium, Rhizomucor, Mucor, Rhizopus, Lichtheimia* and *Galactomyces.* Each of these fungi able to enhance the production of specific enzymes that is beneficial in biotechnology studies. Therefore, fungi isolated from rotten fruit were used in this research to study which species of fungi able produce feruloyl esterase which is essential enzymes required in the production of ferulic acid from agricultural waste.

2.2 Phenolic compound

According to Palacios et al. (2011) phenolic compounds are aromatic hydroxylated compounds that consist of one or more aromatic rings with one or more hydroxyl groups. Phenolic compound are classified into non-soluble compound and soluble compound. Non-soluble compound consist of tannins, lignin and cell wall bound hydroxycinammic acids while soluble compound consist of flavonoid, phenolic acids, phenylpropanoid and quinines (Rispail, Morris, & Webb, 2005). Flavonoid and phenolic acid constitute large and important classes of phenolic compound. Flavonoid is divided into several classes which are flavonols, flavones, flavanols, flavavones, isoflavones and anthocyanidins (Martins et al., 2011). Phenolic acids are divided in two subclasses which are hydroxybenzoic acids and the hydroxycinnamic acids. Hydroxybenzoic acids consists of gallic acid, p-hydroxybenzoic acid, protocatechuic acid, vanillic acid, ellagic acid, gentisic acid, salicylic acid and syringic acid, while hydroxycinnamic acids consists of caffeic acid, ferulic acid, p-coumaric acid, quinic acid, cinnamic acid and chlorogenic acid and sinapic acid (Martins et al., 2011).

2.3 Ferulic acid

Ferulic acid is a hydroxycinnamic acid derived from phytochemical phenolic compounds. Ferulic acid play an important role in prevention of cancers, inhibition of brain damage by Alzheimer's proteins, preventing prostate growth, inhibition of diabetes-induced free radical development, dropping cholesterol manufacture and stimulation of the immune system. It can be extracted from natural resources by three types of pathways which are from low-molecular weight ferulic conjugates, from plant cell walls and from tissue culture or microbial fermentation. Plant cell walls composed of polysaccharides which form a

complex with polymers, such as lignin and cellulose. Ferulic acids are cross-links with polysaccharides through ester linkages. Recently, enzymatic degradation of cell wall polysaccharides using feruloyl esterase was used to break the cross-link and release ferulic acid from plant cell wall constituents (Fazary & Ju, 2007). According to Ralet et al. (1994), the amount of ferulic acid released was correlated by the increases in microbial and enzymic degradations cell wall polysaccharides.

2.4 Feruloyl esterase

Feruloyl esterase is the enzymes responsible for cleaving the ester link between polysaccharides and monomeric or dimeric ferulic acid. This enzyme activity liberates phenolic acids such as ferulic and p-coumaric acid. Feruloyl esterase play a significant role in retrieving phenolic compounds from agricultural waste such as wheat straw, rice straw, and sugarcane bagasse. These enzymes can be produced by different types of fungi (Ou et al., 2011). Recently, *Aspergillus niger* strain I-1472 was shown to produce polysaccharide-degrading enzymes which are feruloyl esterase that released ferulic acid from natural feruloylated oligosaccharides (Fazary & Ju, 2007). Ferulic acid is link to hemicellulose and lignin. Feruloyl esterase play an important role in the degradation of the complex structure of the plant cell wall by hydrolyzing the ferulate ester groups involved in the cross-linking between hemicelluloses and between hemicellulose and lignin (Fazary&Ju, 2007). Degradation of the complex structure of the plant cell wall releases ferulic acid. The ferulic acid released can be converted into valuable molecules such as polymers, epoxides, vanillic acid derivatives, catechol, and vanillin (Ou et al., 2011).

2.5 Solid state fermentation

Solid-state fermentation (SSF) is a process of fermenting solids materials without the presence of free water but enough moisture is required by the substrate to support growth and metabolism of microorganism. Fungi are the most suitable microorganism used in SSF because it grows in nature on solid substrates. SSF bring cultivated fungi in tight contact with the substrates and helps to accomplish the highest nutrient concentration from the substrate for fermentation (Bhargav et al., 2008). According to Mienda et al. (2011), low moisture content of SSF is suitable condition for fungi to growth. Besides, it also increases the production of compound and decreases the possibilities of contamination by yeast and bacteria. Recently, great attention has been given to agricultural waste such as bagasse, husk, cobs and straw that are generated every year as they can be used as solid substrates in SSF processes for the production of different bioactive phenolic compounds. All of these agricultural residues are abundantly available and cheap renewable feedstock for the production of value-added compounds (Martins et al., 2011). In industrial level, SSF are the most suitable techniques use for the production of valuable compound because it only need low energy requirement and able to decrease the production costs (Mienda et al., 2011).

2.6 Agricultural waste

Agricultural waste can be used as substrates for the production of enzymes, organic acids, antibiotics, flavor and aroma compounds, and bioactive compounds by solid state fermentation (SSF) (Martins et al., 2011). Agricultural wastes such as straw, bagasse, cobs, and husks are lignocellulosic materials composed of three important constituents which are cellulose, hemicellulose and lignin. Various phenolic compounds such as ferulic, p-coumaric, syringic, vanillic and p-hydroxybenzoic can be found in lignin segment of

lignocellulosic materials. Agricultural wastes can be used as carbon sources for the fungi in SSF. Fungi grown on agricultural residues and they utilize the polysaccharides after lignin degradation in order to grow and reproduce (Martins et al., 2011). Fermentation of agricultural waste by fungi during SSF is able to improve their phenolic content and antioxidant activity. In recent studies, wheat grain fermented by *Aspergillus oryzae* and *Aspergillus awamo*ri in SSF has higher phenolic content and antioxidant properties (Martins et al., 2011). There are two types of agricultural waste that have been used as substrates in this research. They are rice husk and empty fruit bunch.

2.6.1 Empty fruit bunch

Malaysia is one of the major palm oil producers in the world. About 17.6 million tonnes of oil palm empty fruit bunch (OPEFB) was generated at the mills in 2005 (Ariffin et al., 2008). Hamzah, Idris, and Shuan (2011) found that OPEFB contain 44.2% of cellulose, 33.5% of hemicelluloses and 20.4% lignin. This agricultural waste are suitable substrate that can be used in this experiment because of they are cheap and easily to obtain due to their abundant production per year.

2.6.2 Rice husk

Rice is the one of major food crop in Asia and Africa. Yearly production of rice is about 600 million tons worldwide (Kumar et al., 2012). Rice husk is a by-product of rice production accounts for 23% of total paddy weight (Frimpong–Mansoet al., 2011). Kumar et al. (2012) stated that there about 120 million tons of rice husk are produced each year in the world. According to Soltani et al. (2015), rice husk is a lignocelluloses material which contains 34.4% of cellulose, 24.3% of hemicellulose and 19.2% of lignin. Due to their large annual production and cost effective, rice husk can be used in this experiment as a substrate.

3.0 MATERIAL AND METHOD

3.1 Isolation

The rotten fruit was surface sterilized with cotton wool soaked in 70% alcohol and was cut into small sections (3mm diameter) using sterilized knife or scalpel (Tafinta et al., 2013). Small section of the rotten fruit were inoculated onto Potato Dextrose Agar (PDA) containing chloramphenicol (30 mg/l) and incubated at 28°C for 7 days (Alwakeel, 2013). Any species of fungi grown on the PDA plates was sub-cultured subsequently and the pure culture of fungi was obtained (Siripong et al., 2009).

3.2 Screening of phenolic producing fungi

3.2.1 Total phenolic content

The method is adopted from Amin et al. (as cited in Azlim et al., 2010). The amount of total phenolic compound in extracts was determined with the Folin- Ciocalteu reagent. Gallic acid was used as a standard and the total phenolics were expressed as mg/g gallic acid equivalents. The 0.5 mg/mL stock standard solution of gallic acid was prepared by dissolving 250 mg of dry gallic acid in 1 mL of extracting solvent. Then, they were diluted to 500 mL of distilled water and then the stock solution was stored at 4°C. Concentration of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/mL of gallic acid were prepared in methanol. Concentration of 0.1 and 1 mg/mL of plant extract were also prepared in methanol and 100 µL of each sample were transferred into microcentrifuge tube. 0.75 mL of Folin- Ciocalteu reagent was added into the tube and the mixture was placed at room temperature for 5 min. Then, 0.75 mL of 6% sodium carbonate were added into the tubes. The mixture will be placed at room temperature for 90 min and then the absorbance will be read at 725 nm using spectrophotometer. The result obtained was recorded.

3.2.2 Feruloyl Esterase (FAE) assays

The method is adopted from Ou et al. (2011) with a slight modification. Feruloyl esterase activity was assayed by analysis of free ferulic acid released from de-starched rice dusk (DSRH). The reaction mixture contained 100 mg of DSRH and 2.5 mL of enzyme in 2.5 mL phosphate buffer in a final volume of 5.0 mL and was heated in water bath with temperature 40 °C for 4 hours. The reaction was stopped by putting the mixture into boiling water for 5 minutes. Then the mixture was centrifuge with 10000 rpm for 5 minutes and the absorbance was read at 410 nm. Feruloyl esterase activity (1 U) was defined as the enzyme produced by 1 g of carbon source that released 1 μmol ferulic acid per minutes.

3.3 Morphological characterization

The morphological features such as colony growth pattern, conidial morphology, pigmentation, growth of spore, and other special features of the fungal isolates was determined by macroscopic and microscopic technique. The fungus was re-cultured from pure cultures for 2-3 days. A small segment of mycelium from the typical culture was picked using a sterile inoculating needle and then was place onto a slide containing lactophenol blue. The slide was examined under the light microscope with x10, x40 and x100 objective lens to identify the growth of spore, hyphae and other special structures of fungi.

3.4 Molecular identification

3.4.1 DNA extraction

Fungal DNA extraction is adopted from a method conducted by Cubero et al. (1999). Fungi were inoculated in conical flask containing 25 mL of Potato dextrose broth supplemented with chloramphenicol. Then, the fungi were incubated for 6-7 days. The fungi were filtered by using filter paper and the fungal met was place into mortar. Liquid nitrogen was poured and the frozen fungal met was grind quickly into powder. The fungal powder was transferred into 500 µL of extraction buffer (1% w/v CTAB; 1M NaC1;100 mM Tris; 20 mM EDTA; 1% w/v polyvinyl polypyrolidone, PVPP) in 1.5mL microcentrifuge tube and incubated for 30 min at 70 °C in water bath. 500 µL of chloroform: isoamyl alcohol (24:1) was added and mixed. The mixture was centrifuged at 10000 g for 5 minutes at room temperatures. The upper aqueous phase was transferred into new tube and two volumes of precipitation buffer (1% w/v CTAB; 50 mM Tris-HC1; 10 mM EDTA; 40 mM NaC1) were added to the supernatant and mixed well by inversion for 2 min. The mixture was centrifuged for 15 min at 13000 g at room temperature and the pellet was collected. The pellet was resuspended in 350 µL of 1.2 M NaCl, to which one volume of chloroform:isoamyl alcohol (24:1) was added. This was mixed vigorously and centrifuged for 5 min at 10000 g at room temperature. The upper phase was transferred into a new tube and 0.6 volume of isopropanol was added. This was mixed by inversion and the tube was placed at -20 °C for 15 min. The final pellet was collected by centrifugation for 20 min at 13000 g at 4 °C and then washed with 1 mL of 70% ethanol and recollected by centrifugation for 3 min at 13000 g at 4°C. The pellet was drained and dried at 50°C and resuspended in 10-25 µL TE buffer (10 mM Tris pH 7.4, 1 mM EDTA).

3.4.2 Polymerase chain reaction (PCR)

The method of Ferrer et al. (2001) was adopted. Two types of universal primer which are ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') were used for fungal amplification. The 26 µL PCR mixtures were prepared by the mixing of 3 µL of DNA template, 2 µL of $MgCl_2$, 2.55 µL of PCR buffer, 1 µL dNTPs, 1 µL of forward and reverse primer, and 0.5 µL of Taq DNA polymerase. The amplification was started with 1 cycle at 95°C for 5 min, following by 35 cycles with a denaturation step at 95°C for 30 s, an annealing step at 55°C for 1 min, and an extension step at 72°C for 1 min, following by 1 cycle at 72°C for 6 min. PCR products were separated by electrophoresis in 2% agarose gels in Tris-acetate- EDTA buffer and stained with ethidium bromide (Ferrer et al., 2001).

3.4.3 Analysis of the amplified products and sequencing

The method of Ferrer et al., (2001) was adopted. 4 µL aliquots of each amplified product were electrophoretically separated in a 2% agarose gel in 1 x Trisborate-EDTA buffer and were visualized using ethidium bromide under UV illumination. The PCR product was gel purified and sequenced.

3.5 Solid state fermentation of rice husk and empty fruit bunch

The method is adopted from Ibrahim et al. (2012). The selected fungi were grown on Potato Dextrose Agar (PDA) plates until sporulation (4-6 days) at 30°C. Approximately, 5 mL of sterile distilled water which contain 0.1% (v/v) Tween 80 were added into the agar plate and was shaking vigorously. The spore suspension was modified to the spore concentration of $6x10^6$spores/mL. Five grams of dry rice husk were dried under sunlight

and grind into 0.75 mm particle size of substrate. Once prepared, the 5 g of rice husk were autoclaved at 121±1 °C for 45 min and was inoculated with spore suspension with concentration of $6x10^6$spores/mL. The initial moisture was adjusted to 70% (w/v) and was incubated at 30°C in 12 days. The enzymes produced were harvested every two days. This process was repeated using empty fruit bunch.

3.6 Extraction and analysis of phenolic compound

A mass weighing 5 g of fermented rice husk were harvested by using 20 mL of 60 % extraction buffer. The extract gained was filtered through muslin cloth into a tube and centrifuged at 10000 rpm for 5 min. The supernatant were collected and proceed with total phenolic content and feruloyl esterase assay.